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## The in vitro addition of docosahexaenoic acid (DHA) improves the quality of cooled but not frozen-thawed stallion semen

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1 **The *in vitro* addition of docosahexaenoic acid (DHA) improves the quality of cooled**  
2 **but not frozen-thawed stallion semen**

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4 *In vitro* addition of DHA to stallion semen

5  
6 D.M. Silva<sup>A,B,C</sup>, S.A. Holden<sup>B</sup>, A. Lyons<sup>B</sup>, J.C. Souza<sup>C</sup>, S. Fair<sup>B,D</sup>

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8 <sup>A</sup>*Instituto Federal de Educação, Ciência e Tecnologia do Sul de Minas Gerais –*  
9 *Campus Machado, Machado, Minas Gerais, Brazil*

10 <sup>B</sup>*Laboratory of Animal Reproduction, Department of Life Sciences, Faculty of Science*  
11 *and Engineering, University of Limerick, Castletroy, Co Limerick, Ireland*

12 <sup>C</sup>*Department of Animal Science, Universidade Federal de Lavras, Lavras, Minas*  
13 *Gerais, Brazil*

14 <sup>D</sup>*sean.fair@ul.ie*

15  
16 **ABSTRACT**

17  
18 The aim of this study was to assess the effect of the addition of docosahexanoic acid  
19 (DHA) on the *in vitro* quality of cooled and frozen-thawed stallion semen. In  
20 Experiment 1, semen from 10 stallions was collected (3 ejaculates per stallion). Semen  
21 was diluted to  $100 \times 10^6$  spermatozoa/mL with 0.02 mM of vitamin E (VE) and 0, 1, 10  
22 or 20 ng of DHA/mL and frozen. Semen was thawed and total motility (TM), acrosome  
23 integrity and morphology were assessed. In Experiment 2, semen from 3 stallions was  
24 collected (3 ejaculates per stallion) and frozen as in Experiment 1, but VE and DHA  
25 were added after thawing. Total motility and progressive linear motility (PLM) were  
26 assessed at 30, 60 and 120 min and viability, acrosome integrity and membrane fluidity  
27 at 30 min. In Experiment 3, semen from 5 stallions was collected (1-3 ejaculates per  
28 stallion), diluted to  $20 \times 10^6$  spermatozoa/mL and stored at 4°C. After 1, 24, 48 and 72  
29 h, TM, PLM, viability, membrane fluidity and lipid peroxidation were assessed. The  
30 addition of DHA had no effect on frozen semen (Experiments 1 and 2) but improved  
31 TM, PLM and membrane fluidity in cooled stallion semen.

32  
33 **Keywords:** Equine, Sperm, PUFA, Fertility

34

## 35 **1. Introduction**

36

37 Artificial insemination with cooled and frozen-thawed semen is widely used in  
38 the equine sporthorse breeding industry (Aurich and Aurich, 2006). To achieve  
39 acceptable pregnancy rates with cooled stallion semen it must be inseminated within 24-  
40 36 h of semen collection (Lindahl et al., 2012) compared to 3 days with liquid bull  
41 semen (Murphy et al., 2015) and 5 days with boar semen (Johnson et al., 2000). The  
42 pregnancy rates achieved following the use of frozen-thawed stallion semen are lower  
43 (approximately 45%; Miller, 2008) than those in cattle (about 54%; Odhiambo et al.,  
44 2014) but are also highly dependent on the individual stallion (Haadem et al., 2015).

45 During temperature decreases stallion spermatozoa undergo a membrane lipid  
46 phase change, in which it transitions from a liquid to a gel phase with peak phospholipid  
47 transition thought to occur in stallion spermatozoa at approximately 20°C (Parks and  
48 Lynch, 1992). The cholesterol/phospholipid ratio is thought to influence spermatozoa  
49 membrane fluidity (Klein et al., 1995) and affect the stability of the membrane during  
50 spermatozoa cooling, freezing and subsequent re-thawing (Darin-Bennett and White,  
51 1977). Thus intact lipid molecules are necessary for a good functional spermatozoa  
52 membrane (Bustamante-Filho et al., 2014) which can survive the freeze-thaw process.  
53 In addition, there are differences in the lipid composition of the plasma membrane  
54 between different stallions, which may help explain the variations in spermatozoa  
55 resistance to the cooling and as well as the freeze-thaw process (Battelier et al., 2001).  
56 The phospholipid profile of the stallion spermatozoa plasma membrane is similar to that  
57 of the boar. It contains high levels of docosapentaenoic acid (DPA), an omega-6  
58 polyunsaturated fatty acid (PUFA), and docosahexaenoic acid (DHA), an omega-3-  
59 PUFA (Parks and Lynch, 1992). While it may be desirable to increase the omega-3-  
60 PUFA content of spermatozoa using dietary or *in vitro* supplementation, so as to  
61 increase membrane fluidity, this strategy may also promote susceptibility of the  
62 spermatozoon to lipid peroxidation resulting in membrane damage (Schmid-Lausigk  
63 and Aurich, 2014). Thus, beneficial and detrimental effects of PUFAs enrichment are  
64 closely balanced in stallions (and other species) due to sensitivity to reactive oxygen  
65 species (ROS; Pena et al., 2011). The relationship between lipid profile to the plasma  
66 membrane and spermatozoa quality in stallions remains poorly understood. In contrast,  
67 this relationship has been studied in humans (Lewis, 2007; Aitken et al., 2012), bulls

68 (Rodrigues et al., 2015) and boars (Chung et al., 2015; Barranco et al., 2015; Radomil et  
69 al., 2011).

70 A number of studies supplementing PUFA's in the diet have demonstrated a  
71 beneficial effect on semen quantity and quality in bulls (Gürler et al., 2015; Moallem et  
72 al., 2015), boars (Liu et al., 2015) and rams (Fair et al., 2014). Brinsko et al., (2005)  
73 reported beneficial effects of dietary supplementation of stallions with a DHA  
74 nutraceutical on both frozen-thawed and cooled semen quality, with the most notable  
75 improvements in stallions whose semen did not tolerate cooling well. In an attempt to  
76 compensate for the damages caused to stallion spermatozoa during storage, researchers  
77 have studied the effect of the addition of substances to semen during processing. A  
78 number of studies have shown that the *in vitro* addition of fatty acids to semen  
79 improved the quality of cryopreserved (Büyükleblebici et al., 2014; Kaka et al., 2015a;  
80 Sampaio et al.) and liquid stored bull semen (Kiernan et al., 2013) as well as  
81 cryopreserved boar semen (Chanapiwat et al., 2009) but there is no published study on  
82 the effect of the exogenous DHA addition to stallion semen.

83 Thus, the objective of this study was to assess the effect of the *in vitro* addition  
84 of DHA to stallion semen before freezing, after thawing and before cooling on a range  
85 of *in vitro* spermatozoa quality parameters.

86

## 87 **2. Materials and method**

88

### 89 2.1 Experimental design

90

#### 91 *Animal ethics*

92 All experiments were performed according to appropriate ethical and legal  
93 standard under the approval number: 2014\_11\_11\_ULAEC (University of Limerick,  
94 Ireland).

95

#### 96 *Experiment 1: Effect of the addition of docosahexaenoic acid to stallion semen* 97 *prior to freezing*

98 The aim of this experiment was to assess the effect of the addition of DHA (cis-  
99 4,7,10,13,16,19-Docosahexaenoic acid, Sigma, Arklow, Ireland, 25 mg) and vitamin E  
100 (VE;  $\alpha$ -Tocopherol, Arklow, Ireland, Sigma) to semen before freezing. Semen from 10  
101 Irish Sport Horse stallions of proven fertility, ranging between 13 and 28 years of age,

102 was collected at a commercial stud in Ireland using an artificial vagina (3 ejaculates  
103 from each stallion with a rest interval of at least 3 days between ejaculates) and all  
104 ejaculates were processed individually. Following collection, the gel fraction was  
105 removed following which total motility (TM) was assessed subjectively using a phase  
106 contrast microscope (minimum TM of 70% was used; results not presented). The  
107 ejaculate was diluted in a 1:1 ratio of INRA 96 extender (IMV Technologies, L'Aigle,  
108 France) and centrifuged at 600 g for 10 min at 32°C following which the concentration  
109 of the spermatozoa in the pellet was assessed using a photometer (SDM6, Minitube,  
110 Tiefenbach, Germany). The pellet was diluted to  $100 \times 10^6$  spermatozoa/mL in Gent  
111 freezing extender (Minitube), in the presence of (i) 0 ng of DHA/mL + 0.02 mM of VE  
112 (control; T0<sub>VE</sub>), (ii) 1 ng of DHA/mL + 0.02 mM of VE (T1<sub>VE</sub>), (iii) 10 ng of DHA/mL  
113 + 0.02 mM of VE (T10<sub>VE</sub>) or (iv) 20 ng of DHA/mL + 0.02 mM of VE (T20<sub>VE</sub>). The  
114 concentrations of DHA and VE were adapted from Nasiri et al. (2012) as this study  
115 demonstrated a positive effect on spermatozoa characteristics following the addition of  
116 0.02 mM of VE and 10 ng of DHA/mL to frozen-thawed bull semen. The diluted semen  
117 was cooled slowly to 4°C over 60 min and then packaged into 0.5 mL straws (Minitube)  
118 and sealed using polyvinyl alcohol (PVA) powder (Minitube). Straws were frozen to -  
119 110°C (13.9°C/min) in a programmable freezer (IceCube 14S, Minitube, Germany)  
120 following which they were plunged into liquid nitrogen at -196°C. The sperm  
121 concentration within straws was confirmed using a haemocytometer and was  $\pm 10\%$  of  
122 the target concentration.

123 One straw of each treatment was thawed at 37°C for 30 sec (10 stallions with 3  
124 ejaculates per stallion = 30 ejaculates) and maintained at 32°C in a heated-block until  
125 TM and kinematic parameters were analysed using computer assisted sperm analysis  
126 (CASA). A further two straws of each treatment were thawed (5 stallions with 3  
127 ejaculates per stallion = 15 ejaculates) and assessed for acrosome integrity and  
128 membrane fluidity using flow cytometry. Another straw of each treatment was thawed  
129 (5 stallions with 3 ejaculates per stallion = 15 ejaculates) and assessed for morphology.

130

131 *Experiment 2: Effect of the addition of docosahexaenoic acid to stallion semen*  
132 *after thawing*

133 The aim of this experiment was to assess the effect of the addition of DHA to  
134 semen after thawing. Semen from 3 Irish Sport Horse stallions of proven fertility,  
135 ranging between 7 and 17 years of age, was collected at a commercial stud in Ireland

136 using an artificial vagina (3 ejaculates from each stallion), processed and frozen as per  
137 Experiment 1 but without the addition of DHA or VE. Two straws were thawed per  
138 ejaculate at 37°C for 30 sec and semen was diluted to a final concentration of  $25 \times 10^6$   
139 spermatozoa/mL in INRA 96 containing the following: (i) 0 ng of DHA/mL (control;  
140 T0), (ii) control + 0.02 mM of VE (T0<sub>VE</sub>), (iii) 1 ng of DHA/mL + 0.02 mM of VE  
141 (T1<sub>VE</sub>), (iv) 10 ng of DHA/mL + 0.02 mM of VE (T10<sub>VE</sub>) and (v) 20 ng of DHA/mL +  
142 0.02 mM of VE (T20<sub>VE</sub>). All treatments were maintained at 32°C until analysis were  
143 completed (3 stallions with 3 ejaculates per stallion = 9 ejaculates). Total motility,  
144 progressive lineat motility (PLM) and kinematic parameters were assessed at 30, 60 and  
145 120 min following the addition of DHA using CASA (as per Experiment 1) while  
146 viability, acrosome integrity and membrane fluidity were assessed at 30 min following  
147 the addition of DHA using flow cytometry.

148

149 *Experiment 3: Effect of the addition of docosahexaenoic acid to stallion semen*  
150 *prior to cooling*

151 The aim of this experiment was to assess the effect of the addition of DHA to  
152 semen before cooling. Semen from 5 Irish Sport Horse stallions of proven fertility,  
153 ranging between 7 and 20 years of age, were collected, diluted and centrifuged as per  
154 Experiment 2. The pellet was resuspended to  $20 \times 10^6$  spermatozoa/mL in INRA 96  
155 using the same treatments as per Experiment 2. Semen was maintained at 15°C for 2 h,  
156 packaged in 0.5 mL straws, sealed using PVA powder and following a gradual  
157 temperature reduction were stored at 4°C. After 1 h (Day 0), one straw from each  
158 treatment was warmed to 32°C, following which motility (TM and PLM) and kinematic  
159 parameters were assessed (5 stallions with 1 to 3 ejaculates per stallion = 12 ejaculates)  
160 using CASA (as per Experiment 1). Another straw from each treatment was warmed to  
161 32°C following which viability, membrane fluidity and lipid peroxidation were assessed  
162 (3 stallions with 3 ejaculates per stallion = 9 ejaculates) after 24 (Day 1), 48 (Day 2) and  
163 72 h (Day 3) using flow cytometry.

164

## 165 2.2 Sperm Functional Assessments

166

167 *Computer assisted sperm analysis*

168 Total motility and kinematic parameters were analysed using negative phase  
169 contrast (100X) brightfield microscopy on an Olympus BX60 fitted with a CASA

170 system (Spermatozoa Class Analyser, SCA, Microptic, Viladomat, Barcelona, Spain). A  
171 drop (5  $\mu$ L) of diluted semen was placed on a pre-warmed chamber (37°C; Leja  
172 counting chambers; Microptic) and analysed for spermatozoa motion and kinematic  
173 characteristics immediately post-thaw using SCA Evolution software pre-set to record  
174 stallion parameters (SCA Evolution, Microptic, Viladomat, Barcelona, Spain). A  
175 minimum of five microscopic fields with at least 100 spermatozoa were analysed in  
176 each sample using a phase-contrast microscope at 100X fitted with a pre-warmed stage  
177 at 37°C. Objects incorrectly identified as spermatozoa were edited out using the  
178 playback function. The CASA derived motility and kinematic characteristics assessed  
179 were TM, PLM, average path velocity (VAP above 10  $\mu$ m/s), straight line velocity  
180 (VSL), curvilinear velocity (VCL), linearity (LIN), straightness (STR), amplitude of  
181 lateral head displacement (ALH) and beat cross frequency (BCF). None of the  
182 treatments in all three experiments significantly affected any of the kinematic  
183 parameters and therefore these results are not presented.

184

#### 185 *Morphology*

186 Spermatozoa were fixed with 0.2% glutaraldehyde following which 10  $\mu$ L of the  
187 solution was placed on a slide and covered with a coverslip. After placing a drop of  
188 immersion oil on the coverslip, percentage of morphologically normal and abnormal  
189 spermatozoa was assessed using a phase-contrast microscope at 1000X. At least 100  
190 spermatozoa were assessed in each sample.

191

#### 192 *Assessment of acrosome integrity, membrane fluidity, viability and lipid* 193 *peroxidation*

194 Samples were diluted using phosphate buffered saline (PBS) medium to a  
195 concentration of  $6 \times 10^6$  spermatozoa/mL and were analysed using a flow cytometer  
196 (Guava EasyCyte 6HT-2L, Merck Millipore, Billerica, USA) equipped with both a  
197 krypton (640 nm) and an argon (488 nm) laser. Appropriate single colour controls were  
198 prepared to establish the respective fluorescent peaks of the individual stains. These  
199 were used in conjunction with the forward scatter (FSC) and side scatter (SSC) signals  
200 to discriminate spermatozoa from debris. Fluorescent events were recorded using  
201 GuavaSoft (Version 2.7, Merck Millipore) and all variables were assessed using  
202 logarithmic amplification. In each sample 10,000 gated events were captured.

203

204           Acrosome integrity was assessed using a method adapted from Murphy et al.  
205 (2015). Briefly the fluorescent stains Alexa Fluor 647 PNA (AF647; lectin peanut  
206 agglutinin from *Arachis hypogaea*; Ex/Em: 650/688; Life Technologies) was added to a  
207 final concentration of 6 µg/mL and was incubated at 32°C in the dark for 15 min.  
208 AF647 fluoresces in the presence of the enzyme acrosin, which is exposed upon the loss  
209 of the acrosomal cap. SYTO 16 (Ex/Em: 488/518 nm; Life Technologies, Carlsbad,  
210 USA) was added to the sample at a final concentration of 100 nM and incubated in the  
211 dark at 32°C for 15 min. SYTO 16 works by binding to nucleic acids. Following this  
212 incubation period the fluorescent stain propidium iodide (PI; Ex/Em: 535/617 nm; Life  
213 Technologies) was added to the sample at a final concentration of 15 µM and incubated  
214 for further 15 min. Since PI can not permeate live cells it is used to detect dead cells, PI  
215 binds to DNA by intercalating between the bases with little or no sequence preference.  
216 Post incubation, samples (200 µL) were transferred to a 96-well microplate and  
217 analysed. The fluorescence of AF647, SYTO 16 and PI was analysed via the  
218 photodetector 661/19, 525/30 and 583/23 nm BP filter, respectively, no compensation was  
219 needed. The percentage of viable spermatozoa with intact acrosomes was calculated as  
220 the percentage of AF647 negative cells of the PI negative population as initially gated  
221 based on controls, FSC and SSC.

222

223           Membrane fluidity was assessed using a method adapted from Murphy et al.  
224 (2014). The apoptotic stain Yo-Pro-1 (Ex/Em: 491/509; Life Technologies) was added to  
225 a final concentration of 50 nM and incubated at 32°C in the dark for 10 min. Yo-Pro-1  
226 works by identifying apoptotic cells. Following the incubation period, to assess  
227 membrane fluidity, the fluorescent probe merocyanine 540 (M540; Ex/Em: 555/576;  
228 Sigma, Wicklow, Ireland) was added to a final concentration of 10 µM and incubated in  
229 the dark for a further 15 min. M540 binds to the surface of polarized membranes and  
230 fluoresces upon membrane depolarisation, thus indicating increased membrane fluidity.  
231 Post incubation, samples (200 µL) were transferred to a 96-well microplate and  
232 analysed. Fluorescence of Yo-Pro-1 and M540 were read with the photodetector  
233 (525/30 nm BP filter). High membrane fluidity was defined as the percentage of viable  
234 cells (Yo-Pro-1 negative) positive for M540.

235

236 Viability was assessed using two fluorescent stains: SYTO 16 and PI using a  
237 method adapted from Murphy et al. (2015). SYTO 16 was added to a final  
238 concentration of 100 nM and incubated at 32°C in the dark for 15 min. Subsequently, PI  
239 was added at a final concentration of 15 µM and incubated for a further 15 min. Post  
240 incubation, samples (200 µL) were transferred to a 96-well microplate (Corning Inc.,  
241 Corning, NY, USA) and analysed. SYTO 16 was read with the photodetector (525/30  
242 nm BP filter) and PI was read with the photodetector (583/23 nm BP filter), no  
243 compensation was needed. The percentage of viable cells was expressed as the  
244 percentage of cells positive for SYTO 16, but negative for PI.

245

246 Lipid peroxidation was assessed using two fluorescent stains: BODIPY C<sub>11</sub>  
247 (Ex/Em: 581/591; Life Technologies) and PI. BODIPY C<sub>11</sub> was added to a final  
248 concentration of 2 nM and incubated at 32°C in the dark for 15 min. BODIPY C<sub>11</sub>  
249 works by detecting ROS in cells and membranes. Following the incubation period, PI  
250 was added to a final concentration of 15 µM and incubated for further 15 min. Post  
251 incubation, samples (200 µL) were transferred to a 96-well microplate and analysed.  
252 Fluorescence of BODIPY C<sub>11</sub> and PI were read with the photodetector (583/23 nm BP  
253 filter). Lipid peroxidation was defined as the percentage of viable cells (PI negative)  
254 positive for BODIPY C<sub>11</sub>.

255

### 256 2.3 Statistical analysis

257

258 Data were examined for normality of distribution, tested for homogeneity of  
259 variance and analysed using an Analysis of Variance (ANOVA; Experiments 1 and 2)  
260 or repeated measures ANOVA (Experiments 2 and 3) in the Statistical Package for the  
261 Social Sciences (SPSS; version 22.0, IBM, Armonk, USA). The final statistical model  
262 employed, included the main effects of treatment, incubation period, stallion and their  
263 interactions. Post hoc tests were conducted using the Tukey test and P<0.05 was  
264 deemed to be statistically significant. All results are reported as the mean ± the standard  
265 error of the mean (s.e.m.).

266

## 267 3. Results

268

269 3.1 Experiment 1: Effect of the addition of docosahexaenoic acid to stallion semen  
270 prior to freezing

271

272 There was no effect of treatment on any of the *in vitro* parameters assessed  
273 ( $P>0.05$ ), with an overall post-thaw TM of  $50.3 \pm 4.02\%$ , percentage of spermatozoa  
274 with intact acrosomes in the live population of  $95.7 \pm 0.73\%$ , percentage of spermatozoa  
275 with high membrane fluidity in the live population of  $31.0 \pm 3.49\%$  and percentage of  
276 spermatozoa with normal morphology of  $74.6 \pm 2.26\%$ . There was effect of stallion  
277 ( $P<0.001$ ) on all motility and flow cytometric parameters assessed, but there was no  
278 treatment by stallion interaction.

279

280 3.2 Experiment 2: Effect of the addition of docosahexaenoic acid to stallion semen  
281 after thawing

282

283 There was no effect of treatment, stallion or their interactions on TM or PLM  
284 ( $P>0.05$ ). Over all the time points assessed (30, 60 and 120 min following the addition  
285 of DHA) TM was  $29.6 \pm 5.07\%$  and PLM was  $14.4 \pm 4.49\%$ . There was an effect of  
286 incubation period with TM and PLM ( $P<0.001$ ) decreasing over time. There was an  
287 interaction between incubation period and stallion in both TM and PLM ( $P<0.001$ ).

288 There was no effect of treatment, stallion or their interaction on viability  
289 ( $P>0.05$ ), while there was no effect of treatment or treatment by stallion interaction on  
290 acrosome integrity and membrane fluidity ( $P>0.05$ ). Overall viability was  $30.4 \pm 4.24\%$ ,  
291 percentage of spermatozoa with intact acrosomes in the live population was  $97.6 \pm$   
292  $0.29\%$  and percentage of spermatozoa with high membrane fluidity in the live  
293 population was  $52.9 \pm 4.85\%$ . There was effect of stallion ( $P<0.001$ ) on acrosome  
294 integrity and membrane fluidity.

295

296 3.3 Experiment 3: Effect of the addition of docosahexaenoic acid to stallion semen  
297 prior to cooling

298

299 There was an effect of treatment on TM, with the T20<sub>VE</sub> having greater TM than  
300 the T0 ( $P<0.05$ ; Figure 1). TM declined with day of storage ( $P<0.001$ ) but this was not  
301 affected by treatment ( $P>0.05$ ). There was an effect of stallion on TM ( $P<0.001$ ), but no  
302 stallion by treatment interaction ( $P>0.05$ ). There was an effect of treatment on PLM, with

303 all the DHA treatments having greater PLM than both T0 and T0<sub>VE</sub> (P<0.001; Figure 2).  
304 PLM declined with day of storage (P<0.01) but was not affected by treatment (P>0.05).  
305 There was an effect of stallion on PLM (P<0.01) but no stallion by treatment interaction  
306 (P>0.05).

307 There was no effect of treatment, day of storage or their interaction on viability  
308 (P>0.05), with overall viability (on Days 0 to 3) of 55.2 ± 7.10%. There was an effect of  
309 stallion (P<0.05) but no stallion by treatment interaction (P>0.05).

310 There was an effect of treatment on membrane fluidity (P<0.001), with T10<sub>VE</sub>  
311 (28.7 ± 4.35%) and T20<sub>VE</sub> (29.4 ± 5.18%) having a greater percentage of spermatozoa  
312 with high membrane fluidity in the live population than the other treatments (19.0 ±  
313 3.85% over Days 0 to 3). There was an effect of day of storage, day by treatment  
314 interaction (P<0.001), stallion and stallion by day interaction (P<0.001) but no  
315 treatment by stallion interaction (P>0.05).

316 There was an effect of treatment on lipid peroxidation (P<0.05), with T0<sub>VE</sub> (13.0  
317 ± 4.40%) having lower lipid peroxidation than T10<sub>VE</sub> (21.3 ± 5.20%). While there was  
318 an effect of day of storage (P<0.001), there was no treatment by day interaction  
319 (P>0.05). There was an effect of stallion (P<0.001) but no stallion by treatment  
320 interaction (P>0.05).

321

#### 322 **4. Discussion**

323

324 This is the first published study to assess the effect of the *in vitro* addition of  
325 exogenous DHA to stallion spermatozoa and has demonstrated beneficial effects on  
326 cooled semen but not when added prior to freezing or after thawing.

327 In contrast to the findings in other species, the addition of DHA to stallion  
328 semen prior to freezing did not affect the spermatozoa quality in any of the parameters  
329 assessed. Increased post-thaw motility (Kaka et al., 2015b; Nasiri et al., 2012; Towhidi  
330 and Parks, 2012), improved morphology, acrosome integrity and membrane integrity  
331 (Kaka et al., 2015b) have been reported after the *in vitro* addition of DHA to bull semen  
332 prior to freezing and it may be as a result of DHA accumulation in the membrane of the  
333 spermatozoon and consequently increased resistance to degradation caused by ice  
334 crystal formation (Nasiri et al., 2012). Others have used a semen extender supplemented  
335 with DHA from fish oil (Kaeoket et al., 2010) or a combination of L-cysteine and DHA-  
336 enriched hen egg yolk (Chanapiwat et al., 2009) prior to freezing boar spermatozoa and

337 reported improved post-thaw motility and acrosomal integrity. It may be that stallion  
338 spermatozoa membranes are more sensitive to the freeze-thaw process than other farm  
339 animal species and the *in vitro* addition of DHA to semen was not enough to promote  
340 improvements in the semen quality. It is plausible that higher concentrations of DHA, or  
341 a more prolonged incubation period, may be required for stallion spermatozoa so as to  
342 avoid the disruption caused by ice crystal formation during the cryopreservation  
343 process.

344         Addition of DHA to stallion semen after thawing did not affect spermatozoa  
345 quality in any parameters analysed. There is a dearth of published studies which added  
346 DHA to semen after thawing. However, after dietary supplementation with a  
347 nutraceutical rich in DHA, improvements in freezability consequently resulting in  
348 increased motion characteristics have been reported in frozen-thawed stallion semen  
349 (Brinsko et al., 2005). It is not known why dietary supplementation of DHA seems to be  
350 more effective in improving frozen-thawed stallion semen quality than *in vitro* addition  
351 of DHA to frozen-thawed stallion semen but it may be due the duration of exposure of  
352 the spermatozoa to DHA or even the low concentrations of exogenous DHA. Future  
353 studies should consider keeping DHA in contact with thawed semen for longer period  
354 than the period used in the present study and/or using higher concentrations of DHA.

355         The improvements observed in the current study in TM, PLM and membrane  
356 fluidity when DHA was added to cooled semen are likely related with the DHA  
357 incorporation into the spermatozoa membrane thus protecting the membrane against the  
358 damage caused due the temperature changes (Nasiri et al., 2012). Therefore, DHA  
359 seems to be important to preserve the membrane functionality when the temperature  
360 change is not severe, from 37°C to 4°C, but the concentrations of DHA used in this  
361 study was not enough to protect the membrane when sub zero temperature were applied  
362 (as in Experiments 1 and 2). In contrast, there was no effect on TM in cooled stallion  
363 semen after dietary supplementation with a DHA-enriched nutraceutical (Brinsko et al.,  
364 2005), this suggests that the *in vitro* addition of DHA to cooled stallion semen is more  
365 efficient in improving TM than supplementation of DHA in the diet.

366         In the current study, the addition of DHA to cooled stallion semen yielded  
367 higher PLM than the controls up to Day 3 of storage, which may be associated with the  
368 capacity of DHA in preventing the disruption of lipid membranes (Meryman, 1966).  
369 Similarly, an improvement in PLM was observed on both Day 1 and 2 of cooled semen  
370 storage when the stallions with <40% initial PLM had their diets supplemented with

371 DHA (Brinsko et al., 2005). On the other hand the *in vitro* addition of exogenous DHA  
372 to cooled bull semen had detrimental effects on PLM and viability (Kiernan et al., 2013)  
373 and may be due to DHA accelerating the production of ROS. Therefore, the use of  
374 antioxidants such as VE along with PUFA supplementation to protect spermatozoa  
375 seems essential.

376 The addition of the exogenous DHA to cooled stallion semen yielded a greater  
377 percentage of spermatozoa with high membrane fluidity and it may be explained by  
378 the several roles of PUFAs, for example their ability to confer upon the spermatozoa  
379 plasma membrane the fluidity it needs to fertilise the oocyte (Whates et al., 2007). The  
380 percentage of spermatozoa with high membrane fluidity decreased from Day 0 to Day 1  
381 which may be due to stallion spermatozoa being susceptible to the drop in temperature,  
382 due to the phase transition of lipids which can damage the plasma membrane (Moran et  
383 al., 1992). After Day 1, spermatozoa with high membrane fluidity survived while  
384 spermatozoa with low membrane fluidity did not, therefore it would appear that the  
385 percentage of spermatozoa with high membrane fluidity in the live population increased  
386 after Day 1.

387 Similarly to this study, Kaka et al. (2015b) found that lipid peroxidation  
388 increased as the DHA concentration added increased. The presence of high levels of  
389 PUFA requires efficient antioxidant levels to protect spermatozoa against lipid  
390 peroxidation (Aitken and Baker, 2004) and it seems that the 0.02 mMol of VE used in  
391 the current study was not sufficient to prevent lipid peroxidation.

392 In conclusion, the present *in vitro* study has demonstrated that the addition of  
393 DHA had a positive effect on the quality of cooled stallion semen, in terms of increased  
394 TM, PLM and percentage of spermatozoa with high membrane fluidity, however, there  
395 was no effect on frozen-thawed semen. A field fertility trial is required to establish if the  
396 *in vitro* addition of DHA to cooled stallion semen can increase *in vivo* fertility.

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#### 398 **Conflict of interest**

399

400 The authors have no conflicts of interest to declare.

401

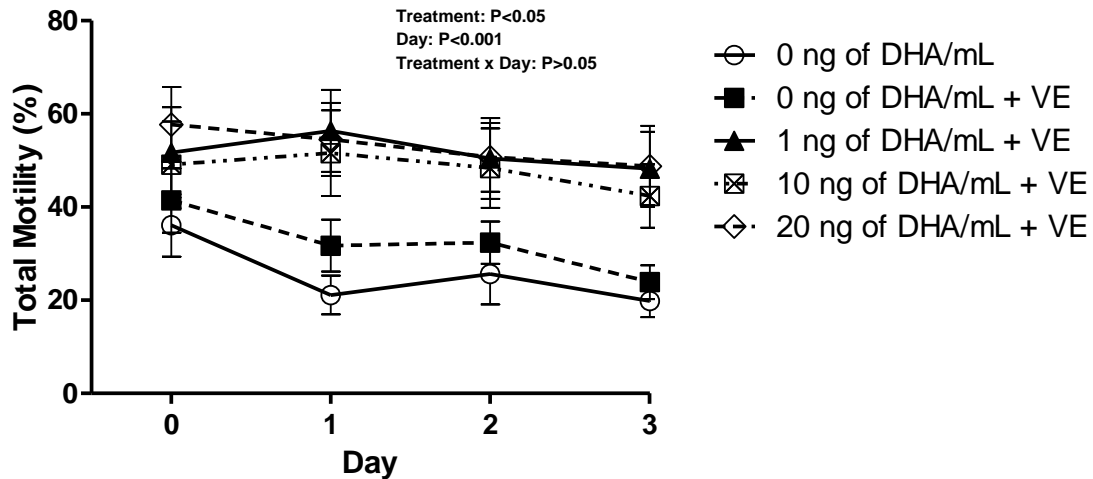
#### 402 **Acknowledgements**

403

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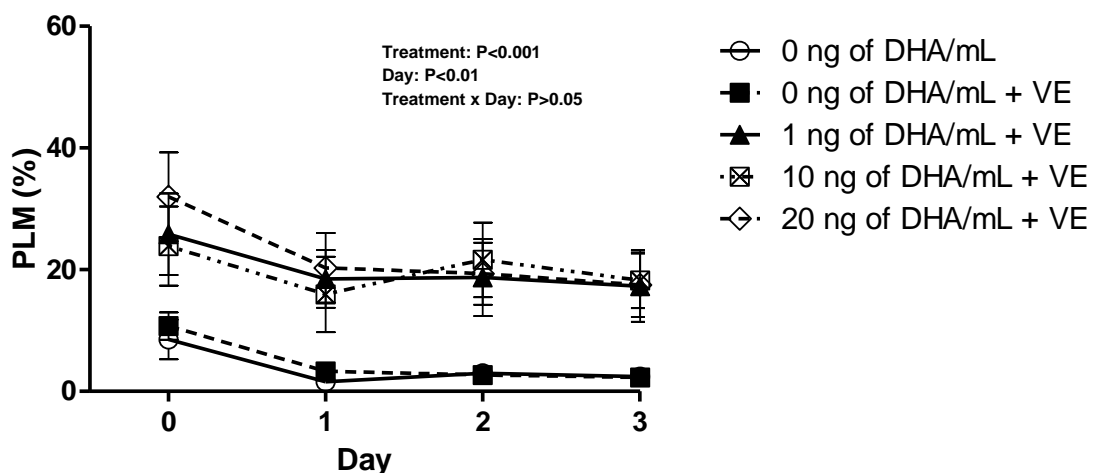
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**Figure 1.** Effect of the addition of docosahexaenoic acid (DHA) in the presence of Vitamin E (VE) prior to cooling to 4°C on total motility of stallion semen (n=5 stallions). Vertical bars represent s.e.m.



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**Figure 2.** Effect of the addition of docosahexaenoic acid (DHA) in the presence of Vitamin E (VE) prior to cooling to 4°C on progressive linear motility (PLM) of stallion semen (n=5 stallions). Vertical bars represent s.e.m.

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